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Using Zebrafish to Study Pathways that Regulate Hematopoietic Stem Cell Self-Renewal and Migration

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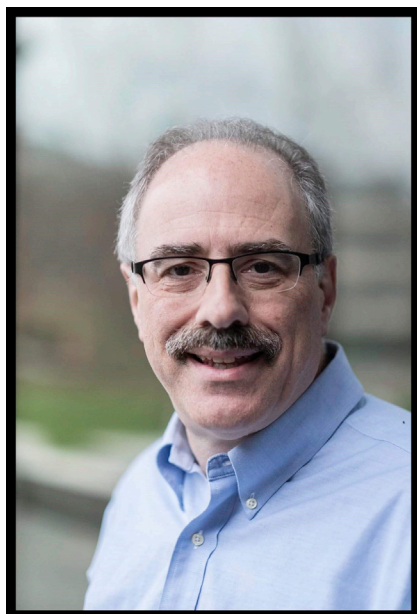
The HSC Niche

The specialized microenvironment that surrounds hematopoietic stem cells (HSCs) is termed as the niche, which is a critical regulator of self-renewal and differentiation of HSCs into blood and immune cell lineages (Orkin and Zon, 2008; Morrison and Scadden, 2014; Boulais and Frenette, 2015). A variety of different cell types, adhesion molecules, and secreted signaling factors are associated with the HSC niche that can directly affect stem cell behavior. Detailed understanding of such HSC-niche interactions is crucial for improving HSC transplantation-based disease therapies. Because of the complex interaction between the niche and HSCs, many aspects of hematopoiesis are difficult to study in cell culture systems. The use of vertebrate model organisms such as mouse, frog, and zebrafish has substantially helped to elucidate pathways of hematopoietic development and regeneration. External development of lower vertebrates such as frog and fish, as well as large numbers of embryos per female, makes many experiments feasible that would not be possible in placental mammals. In the past 25 years we and others have demonstrated that both transcriptional regulation and cellular makeup of the hematopoietic system is largely conserved from fish to humans (Brownlie et al., 1998; Childs et al., 2000; Donovan et al., 2000; Liao et al., 2000; Shafizadeh et al., 2002; Brownlie et al., 2003; Paw et al., 2003; Paffett-Lugassy et al., 2007). After being born from the hemogenic endothelium of the dorsal aorta (DA) (Kissa et al., 2008; Bertrand et al., 2010; Boisset et al., 2010), definitive HSCs enter into blood circulation and then populate an intermediate hematopoietic niche before colonizing the adult marrow. In mammals, this temporary niche is the fetal liver, whereas in zebrafish it is the caudal hematopoietic tissue (CHT), a thin vascular plexus in the tail region of the embryo (Murayama et al., 2006; Orkin and Zon, 2008). Following a rapid expansion, the HSCs will egress the temporary niche to finally colonize the adult marrow, which in mammals are the bones and in zebrafish the kidneys (Traver et al., 2003).

Importance of Niche-Associated Cells in Controlling HSC Fate

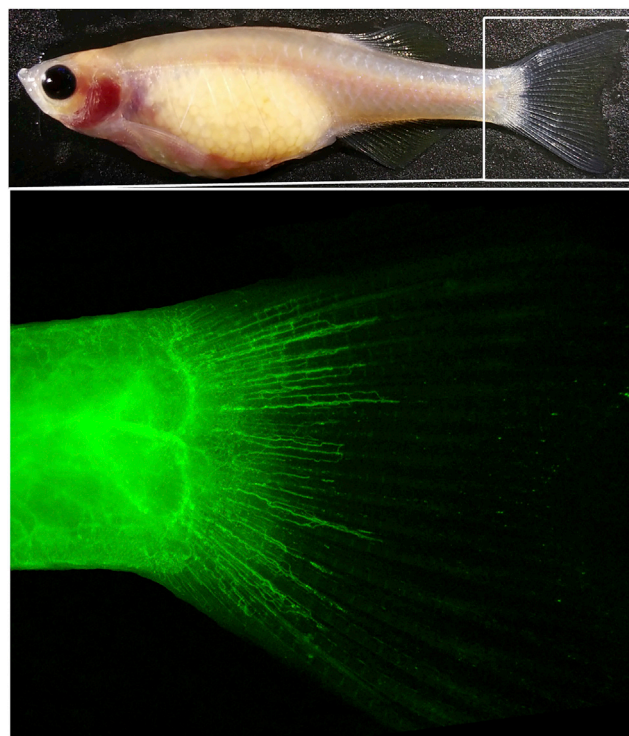
In the adult bone marrow, the sinusoidal vessels form a complex network in close proximity to the HSCs. In such a “perivascular microenvironment” (Kiel et al., 2005; Nombela-Arrieta et al., 2013), endothelial cells (ECs) with distinct properties nurture and expand the hematopoietic stem and progenitor cells (HSPCs). Studies have shown that, in addition to the ECs, many other cell types within the perivascular niche (e.g., stromal cells, sympathetic nerves, osteoblasts) can support HSPCs by supplementing factors including *KIT* ligands and *CXCL12*. Many of these studies relied on visualizing the exogenous HSPCs in a bone explant (Sugiyama et al., 2006; Kohler et al., 2009; Lo Celso et al., 2009; Xie et al., 2009; Mendez-Ferrer et al., 2010; Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013). Directly visualizing the endogenous marrow in a living organism has always been a major challenge in the field for a rigorous analysis of the dynamic cell-cell interactions that are prerequisite to determining the fate and engraftment potential of HSCs.

To this end, our laboratory has utilized the developing zebrafish as a model which, by virtue of being transparent, offers a distinct advantage over the mouse model in visualizing the sequential cell-cell interactions during HSC migration and niche engraftment at a spatiotemporal resolution. By using a regulatory element from the first intron of the mouse *Runx1* locus (+23 kb downstream of the P1 promoter), two transgenic zebrafish lines were generated to drive either *EGFP* (*Runx:GFP*) or *mCherry* (*Runx:mCherry*) to specifically mark the HSCs (Tamplin et al., 2015). HSCs marked by these reporters also express major definitive stem cell markers, such as *cMYB* and *CD41*, in the major embryonic hematopoietic sites, e.g., CHT, DA, kidney, and thymus. The *Runx*⁺ cells are functional, since 3 months post transplantation donor cells produced progeny of all the downstream lineages. Limit dilution assays estimated the stem cell frequency within the *Runx*⁺ cells to be ~1 in 3 in the embryo and 1 in 35 in the adult. Coupled with state-of-the art high-resolution time-lapse microscopy,



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the *Runx*-transgenic lines provided an opportunity to track endogenous HSCs in the live embryo and monitor the dynamic interactions with the niche. By intercrossing *Runx:EGFP* to an *FLK1:mCherry* line (a vascular reporter that marks ECs), we were able to discover striking HSC-EC interactions during HSC travel through circulation to the



Transparent Casper zebrafish allow visualization of blood in the adult animal. GFP reporter for the hemoglobin locus control region (LCR-GFP) is expressed in all zebrafish red blood cells. The inset shows blood circulating through the tail fin.

CHT. As nascent HSCs migrate into CHT, distinct steps of lodgment and niche engraftment can be visualized, beginning with luminal adherence and transendothelial migration. Once in the extravascular space, HSCs interact with the endothelial cells on their abluminal surface. At least five endothelial cells remodel to form a pocket around a specific HSC. In addition, upon intercrossing *Runx:EGFP* and *CXCL12A:dsRedII* (which marks the mesenchymal fibroblasts) transgenic lines, we were able to detect novel HSC-mesenchymal stromal cell interactions, where two stromal cells in close proximity to an HSC oriented the subsequent division plane of the HSC, presumably by determining HSC polarity. Finally, the *Runx:EGFP* transgenic line proved to be an excellent tool for a chemical genetic screen to identify small molecules that modulate the HSC-niche interactions observed in the embryo. This study, apart from validating transforming growth factor β as a negative regulator of HSC proliferation, identified a novel compound, lycorin, which over time strikingly increased the number of HSCs not only in the CHT but also in the kidney marrow of 4-month-old adult fish. This unique study identified novel HSC-niche interactions that lead to long-term changes in the size of the stem cell



pool into adulthood. Currently experiments are investigating the influence of other cell-types in the HSC microenvironment that could potentially alter HSC fate.

Clonality: Establishing and Maintaining an Appropriate Pool of HSCs

The question of how an appropriate pool of HSCs is established and maintained is of both basic and clinical importance. In several blood cancers such as multiple myeloma, acute myeloid leukemia and chronic lymphocytic leukemia somatic mutations have been identified which are thought to lead to an expansion of a malignant clone, often originating from a hematopoietic stem or progenitor cell. However, the same mutations can also be present in healthy, asymptomatic individuals (Jaiswal et al., 2014). Therefore, additional experimental systems are needed to further investigate genetic and epigenetic players driving clonal malignant states. Traditional clonal analysis during native hematopoiesis has been hampered by the fact that being a liquid tissue, spatial information cannot be used to infer lineage relationships. Various innovative approaches have recently given novel insights into the clonality of native hematopoiesis such as using specific HSC promoters (Busch et al., 2015) or utilizing transposon induced DNA barcoding to track hematopoietic clones (Sun et al., 2014). In addition, fluorescent protein expression has been used for clonality studies in a system called Brainbow, originally developed to monitor lineage relationships within the nervous system (Livet et al., 2007). In a Brainbow transgenic animal, there are several cassettes of three genes encoding distinct fluorescent proteins and flanked by different *loxP* sites. Induction of *Cre*-recombinase leads to a random recombination event at any of the *loxP* sites that results in various color combinations in each cell, essentially color-barcoding the cell. *CRE* recombinase can be tissue specific if expressed under specific promoters. Progeny of a color-barcoded hematopoietic cell will have the same color, revealing the clonal nature of a particular lineage. During development in zebrafish, for example, the timing of *CRE* recombinase induction allows one to determine the number of HSC clones in a defined developmental stage by tracking the clonal diversity of adult hematopoietic cells. Using this color-barcoding method, our studies found that there are about 30 HSC clones during their birth in the aorta-gonad-mesonephros (AGM) (Henninger et al., 2017). Furthermore, our system allowed tracking clonal output of HSPCs over time and showed that clonality of peripheral blood remained stable during normal adult hematopoiesis. Stressful stimuli, such as irradiation or transplantation, reduced the number of HSPC clones, likely because of selection that leads to the dominance of one clone over the others. The shift in clonal compositions was apparent by an increased presence of a

color-barcoded hematopoietic output. We are now trying to exploit this system to model malignant clonal hematopoietic states in adult zebrafish by introducing various putative mutations identified from human blood cancers. In comparison with mammalian in vivo models, a greater number of mutational combinations can be tested in a non-transplanted, native hematopoiesis setting. For a typical experiment, we can introduce up to 20 different mutations in about 50 zebrafish, and multiple studies can be run in parallel. A big advantage of the Brainbow system over sequencing-based lineage-tracking approaches is that flow cytometry-based cell sorting allows the isolation of dominant clones. These cells can then be used for further molecular analyses or be subjected to functional testing in transplantation assays. A recent study has used the Brainbow system to track hematopoietic clonal output in the mouse, mostly using transplantations (Yu et al., 2016). Integrating data across various lineage-tracing techniques in both native hematopoiesis and during transplantation will allow us to further unravel mechanisms controlling HSC numbers as well as their dynamics and clonal output.

Chemical Screens: Identifying Conserved Small-Molecule Regulators of HSC Function

Because of their external development, embryos of lower vertebrates such as zebrafish can be easily manipulated. For example, drugs can be added in a precisely timed manner to the media in which the embryos are incubated. The ease by which zebrafish embryos can be treated with chemicals has been utilized in more than 60 phenotypic chemical screens in the past 15 years (Rennekamp and Peterson, 2015). Similar to traditional forward genetic screens in model organisms, a specific phenotype is chosen (e.g., numbers of HSCs in the developing embryo) and a library of chemicals is screened for candidates that can affect this phenotype. Our laboratory alone has conducted around 20 chemical screens, and we believe that the zebrafish is arguably the best model for in vivo phenotypic chemical screening.

A chemical screen targeted at modulators of HSC numbers during embryonic development identified prostaglandin E_2 (PGE_2) a small-lipid mediator (North et al., 2007). Out of a library of around 2,500 chemicals, 12% of all hits were part of the prostaglandin pathway. We further discovered that during adult regeneration, a pulse of PGE_2 conferred an advantage in hematopoietic regenerative capacity in both zebrafish and mice (Goessling et al., 2009; Goessling et al., 2011). Although studies from the 1970s and 1980s had implicated PGE_2 in hematopoiesis and recovery after irradiation, a clear link between HSCs and PGE_2 was missing (Feher and Gidali, 1974; Gentile et al., 1983; Hanson and Ainsworth, 1985). Together with others



we were the first to demonstrate a direct effect of PGE₂ on HSCs, via limit dilution competitive reconstitution assays (North et al., 2007; Hoggatt et al., 2009). Ex vivo treatment of donor bone marrow can increase the number of functional HSCs by 4-fold. Because of promising results in animal models, PGE₂ is now under clinical investigation to improve human HSC transplants (Cutler et al., 2013). This compound also illustrates the strength of starting with in vivo animal models early in the discovery process. PGE₂ was the first compound discovered in a zebrafish chemical screen that entered clinical trials. The transition from the initial zebrafish studies to the human trial took only 2.5 years.

Zebrafish lends itself to chemical screens not only in the embryo but also in the adult. A zebrafish mutant called “Casper” was developed that is completely transparent and allows monitoring of adult tissue in the living organism (White et al., 2008). This fish model permitted tracking and measurement of HSC engraftment after transplantation in an adult live animal (Li et al., 2015). In a competitive transplant experiment we screened 480 chemicals for an advantage in engraftment. We discovered other lipid mediators, epoxyeicosatrienoic acids (EETs), to be potent enhancers of HSC engraftment in zebrafish. Again, the beneficial effect on HSCs was conserved in mouse hematopoietic transplantations. As a member of the eicosanoid family, EETs are related to PGE₂, but the ultimate biochemical pathways regulating synthesis and degradation are distinct. EETs and PGE₂ bind to different receptors (G-protein-coupled receptors [GPCRs]) and elicit distinct immediate downstream signaling. It is intriguing to consider how two lipids through different immediate actions can elicit the same overall phenotype, i.e., a net positive effect on HSC regeneration. PGE₂ is likely acting directly on the HSPCs by enhancing long-term engraftment. It will be interesting to similarly identify a target cellular population of EETs. Determining the receptor for EETs, an as to this date unknown GPCR, will be key for further functional studies. Overall, zebrafish have been successfully used repeatedly as a potent in vivo screening platform to identify chemicals affecting conserved mechanisms of regeneration.

Niche-Associated Signaling Factors that Influence HSC Regeneration and Differentiation

The inflammatory small molecules EETs and PGE₂ identified in our chemical screen are likely also endogenously activated during hematopoietic regeneration. One source of these lipid mediators are niche cells. Our results suggest that the interaction of HSCs and niche cells, such as a rapid remodeling of endothelial cells around a stem cell, may form localized pockets that could effectively increase the concentration of local growth factors and signaling mole-

cules. It is tempting to speculate that PGE₂ and EETs as well as other signaling pathways including bone morphogenetic protein (BMP), WNT, and Notch, exert spatiotemporal control over hematopoietic stem cell self-renewal, differentiation, and regeneration. The hematopoietic system regenerates after sublethal doses of irradiation or after transplantation. During this regeneration, ligands for the developmental signaling pathways and small lipid ligands are upregulated in the stem cell niche, and this facilitates the recovery (Lenox et al., 2005; Congdon et al., 2008; Porter et al., 2013). We have investigated the mechanism of ligand-induced signaling transcription factors on the intrinsic hematopoietic program (Trompouki et al., 2011). Transgenic zebrafish lines that overexpress positive and negative regulators of the WNT and BMP pathway with a heat-shock promoter were subjected to a sublethal dose of irradiation and the recovery was monitored. There was an acceleration of precursor recovery in the presence of WNT or BMP, and treatment of WNT and BMP inhibitors partially blocked recovery. Based on qPCR analysis, higher levels of cell-specific regulators such as *MYB*, *RUNX1*, *LMO2*, and *SCL* occurred with stimulation of the WNT and BMP pathways, providing evidence that the WNT and BMP pathways stimulate the intrinsic hematopoietic program during regeneration.

WNT and BMP signal transduction pathways terminate with specific signaling transcription factors, *TCF7L2* and *SMAD1*, respectively. To understand the targets of WNT and BMP stimulation in hematopoietic cells, chromatin immunoprecipitation sequencing experiments to evaluate DNA binding of *TCF7L2* and *SMAD1* were performed (Trompouki et al., 2011). Both signaling transcription factors bind adjacent to the erythroid-specific transcription regulators *GATA2* and *GATA1* on erythroid-specific genes in erythroleukemic K562 cells; however, in myeloleukemic U937 cells, their binding shifts to myeloid-specific genes in regions that are co-occupied by the myeloid master transcription factor *CEBP-α*. This suggests that the cell-specific transcription factors recruit the signaling transcription factors to local areas of the genome to reinforce the intrinsic hematopoietic program during regeneration, conferring an advantage to the recovering cells. Current experiments are under way to understand the mechanisms that influence recruitment of signaling transcription factors by cell type-specific factors at the critical genomic sites during HSC differentiation.

Many signaling molecules including WNT, BMP, PGE₂, and EET have all been described to play a beneficial role during HSC self-renewal and regeneration (Zon, 2008). Since all of these secreted factors lead to the same outcome—increased regeneration—we are now trying to assess at which point these pathways converge. Initial signaling is different and leads to the activation of distinct



transcription factors. One possible model would be that these transcription factors are recruited to certain important sites called “signaling centers” and then drive a common regenerative gene expression signature. Identifying these putative genomic loci as well as their target genes could reveal fundamental principles governing hematopoietic regeneration. Many open questions remain, such as potential feedback signaling between niche and HSCs, coordination of different temporal kinetics between these pathways, and possible sequential activation of different signaling pathways.

Perspective and Future Implications

Our research strives to elucidate fundamental principles of HSC biology with the potential to have a clinical impact. To unravel the role of the microenvironment for HSCs a direct visualization of *in vivo* HSC behavior would be the most straightforward, yet almost impossible strategy in a mammalian system. Utilizing the transparent zebrafish embryos coupled with a spinning-disk confocal microscope, we are able to visualize the step-by-step events of the HSC-niche interactions *in vivo*. This provides a highest-resolution image of a stem cell niche in any vertebrate species, utilizing electron microscopic resolution of stem cells as they arise and migrate during development. Determining all of the cellular players that constitute the niche and evaluating their behavior in real time is vital for understanding the behavior of regenerative stem cells.

Being able to model development and maintenance of HSC clones throughout an organism's lifespan is critical to testing genetic risk factors affecting their function. Using the Brainbow system, we have developed a novel way to label and track HSC clones in their native state. We believe that the zebrafish Brainbow system is ideal for prospectively introducing mutations into HSPCs and tracking their effect on clonality during native hematopoiesis. We expect that our findings will be also applicable to human hematopoietic malignancies.

Our studies have demonstrated the value of screening and evaluating pathways governing HSC self-renewal/differentiation in the zebrafish system, capitalizing on the excellent genetics and developmental biology of the organism, and utilizing the mouse and human systems to validate our observations and probe mechanism. This approach led to the discovery of PGE₂ and EETs as potent regulators of HSC induction during embryogenesis and engraftment in adults. PGE₂ has shown significant promise to stimulate the engraftment process directly by increasing long-term self-renewal of HSCs, an essential step in successful clinical development. In addition to PGE₂, our strategy has significant potential to identify other small molecules that would improve therapy for patients who have undergone HSC transplantation. Our studies with BMP and

WNT signaling in human erythroid progenitors have documented the surprising co-binding of cell-specific and signaling-induced transcription factors to blood-specific genes, providing a mechanism by which the cell-specific program can be stimulated by developmental growth factors during regeneration. Given our observation that the interaction between master and signaling transcription factors are highly conserved in human, mouse, and zebrafish hematopoietic cells, our approach should be able to manipulate the process for the benefit of treating blood diseases and for improving marrow and cord blood transplantation.

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